

Complete Mineralization of Benzene by Aquifer Microorganisms under Strictly Anaerobic Conditions

ELIZABETH A. EDWARDS* AND DUNJA GRBIĆ-GALIĆ

*Environmental Engineering and Science, Department of Civil Engineering,
Stanford University, Stanford, California 94305-4020*

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Benzene was mineralized to CO₂ by aquifer-derived microorganisms under strictly anaerobic conditions. The degradation occurred in microcosms containing gasoline-contaminated subsurface sediment from Seal Beach, California, and anaerobic, sulfide-reduced defined mineral medium supplemented with 20 mM sulfate. Benzene, at initial concentrations ranging from 40 to 200 μM, was depleted in all microcosms and more than 90% of ¹⁴C-labeled benzene was mineralized to ¹⁴CO₂.

Benzene, a proven human carcinogen (5), is a frequent groundwater contaminant as a result of gasoline and diesel fuel spills into the environment (5, 15, 23). Natural or enhanced biodegradation in situ is a mechanism for remediation of contaminated sites, and benzene and other aromatic hydrocarbons are amenable to bioremediation when oxygen is available or supplied (19). Although benzene is rapidly mineralized in aerobic environments (12, 26), the fate of benzene under anaerobic conditions is not well understood. The anaerobic biodegradation of benzene appears to be mediated by exceptionally fastidious organisms, and conclusive evidence demonstrating complete degradation of benzene in subsurface sediments is lacking. Anaerobic environments contaminated with hydrocarbons are widespread (30), and a fundamental understanding of the processes occurring at these sites is necessary to design effective management and remediation strategies.

The anaerobic degradation of some alkylbenzenes, including toluene and xylenes, is now widely accepted. This degradation has been observed in the field (4, 17, 23, 24) and demonstrated in the laboratory with a variety of different electron acceptors (1, 2, 7–11, 14–16, 18, 20, 31); pure cultures that can grow on toluene in the complete absence of oxygen have been isolated under denitrifying (6, 9, 25) and iron-reducing (21) conditions. In contrast to the numerous studies documenting anaerobic toluene or xylene degradation, only a few studies have presented evidence of benzene transformation under anaerobic conditions. The reported degradation of benzene is slow (28–30) or incomplete (28), and long lag times may precede the onset of degradation (30). One study reported that benzene was degraded under denitrifying conditions, but not under methanogenic conditions (22). In some cases, the possibility that molecular oxygen was involved in the apparent anaerobic degradation of benzene could not be excluded (22, 28). The most detailed study to date demonstrated that benzene was degraded via phenol to methane and carbon dioxide by a mixed methanogenic culture; however, this culture was derived from sewage sludge, not from sediment (13, 27). In this report we present a rigorous demonstration of complete anaerobic mineralization of benzene to carbon dioxide by aquifer-derived microorganisms.

Several studies have shown that when mixtures of ben-

zene, toluene, xylenes, and ethylbenzene are tested simultaneously, there is a sequential anaerobic utilization of the substrate hydrocarbons. Toluene is most often the first hydrocarbon degraded, followed by the isomers of xylene in varying order (1, 2, 7–10, 15, 18). Benzene and ethylbenzene, if degraded at all, tend to be degraded last. We recently studied benzene and alkylbenzene degradation under sulfate-reducing conditions in microcosms containing subsurface sediment from Seal Beach, California, amended with a mixture of benzene and alkylbenzenes (8). We observed that toluene was the first compound to be degraded followed sequentially by *p*-xylene and *o*-xylene. Benzene and ethylbenzene were not degraded. It occurred to us that perhaps the degradation of benzene was prevented not by the lack of suitable organisms but by the presence of other, more available substrates, in this case, toluene and xylenes. Therefore, with the same Seal Beach sediment, six new microcosms were prepared exactly as before (8), except that they were amended with benzene only. Each microcosm contained 100 g of sediment, 160 ml of prereduced defined mineral medium (pH 7; supplemented with 20 mM sulfate), and a 50-ml headspace (80% N₂, 10% CO₂, 10% H₂) in 250-ml bottles sealed with Mininert valves (8). The medium was reduced with amorphous ferrous sulfide (3), and anaerobiosis was maintained throughout as confirmed by the clear color of the resazurin indicator in the medium. One microcosm was sterilized by autoclaving and by the addition of mercuric chloride (30 mg/liter). All microcosms received benzene (99.9%; Sigma Chemical Co., St. Louis, Mo.) at liquid concentrations ranging from 40 to 200 μM. One microcosm (no. 3) also received 140 μM toluene (99.9%; Sigma) as a positive control for the presence of active aromatic hydrocarbon-degrading organisms. These microcosms were incubated statically in the dark inside an anaerobic chamber (Coy Laboratory Products, Grass Lake, Mich.) at room temperature. The concentrations of benzene and toluene were measured by withdrawing 300 μl of headspace from microcosms with a 500-μl gas-tight syringe and injecting this sample into a Carlo Erba Fractovap 2900 Series gas chromatograph (Carlo Erba Strumentazione, Milan, Italy) equipped with a photoionization detector (model PI-52-02, HNU Systems Inc.; 10-eV lamp). Initially, samples were taken once per month until degradation began; after degradation was noted, samples were taken weekly. Methane was measured in 400-μl headspace samples injected onto a Hewlett-Packard 5730A gas chromatograph equipped with a

* Corresponding author.

TABLE 1. Lag times before degradation and initial rates of degradation of benzene in six anaerobic microcosms

Microcosm	Initial benzene concn (μM)	Approx lag time (days)	Initial rate ($\mu\text{M}/\text{day}$) ^a
1	40	30–60	1.0
2	90	30–60	1.9
3 (+ toluene) ^b	90	30–60	0.8
4	140	30–60	3.7
5	200	70–100	0.4
6 (sterile)	90	No degradation	

^a The initial rate was estimated as the slope of the curve of benzene concentration versus time immediately after the onset of degradation.

^b Toluene (140 μM) was added as a positive control for degradation and was completely degraded in less than 30 days.

flame ionization detector. Sulfate was analyzed on a Dionex Series 4000i ion chromatograph, using an electrochemical conductivity detector.

Toluene in the positive control microcosm (no. 3) was completely degraded in less than 30 days. Benzene degradation began in all active microcosms after at least a 30-day lag time (Table 1). The initial rate of benzene degradation increased as initial concentrations increased up to 140 μM . However, at an initial concentration of 200 μM , a longer lag time and much slower rate of degradation were observed, probably as a result of substrate toxicity. The rate of benzene degradation in microcosm 3, which was also amended with toluene, was considerably slower than the rate in a parallel microcosm (no. 2) that did not receive toluene. When the benzene concentration in any microcosm fell below about 1 μM , 3 μl of benzene was added to the microcosm (to a final concentration of 140 μM) from a stock of neat benzene that was kept in the anaerobic chamber. The benzene concentration profiles for microcosms 1 and 6 (sterile control) are shown in Fig. 1.

¹⁴C-benzene (19.3 mCi/mmol; Sigma) was used to confirm the mineralization of benzene. The ¹⁴C activity in the volatile (i.e., benzene), nonvolatile (i.e., cells and nonvolatile intermediates), and CO₂ fractions of samples from ¹⁴C-benzene-amended microcosms was measured as described by Grbić-

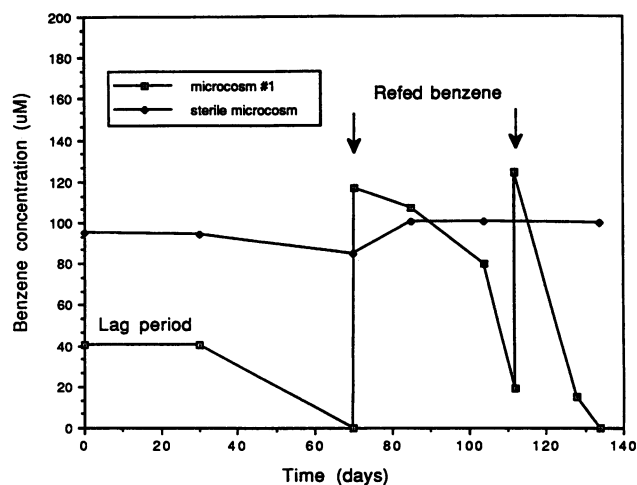


FIG. 1. Benzene degradation in a typical active microcosm compared with a sterile control. The active microcosm was refed benzene on the days indicated by arrows.

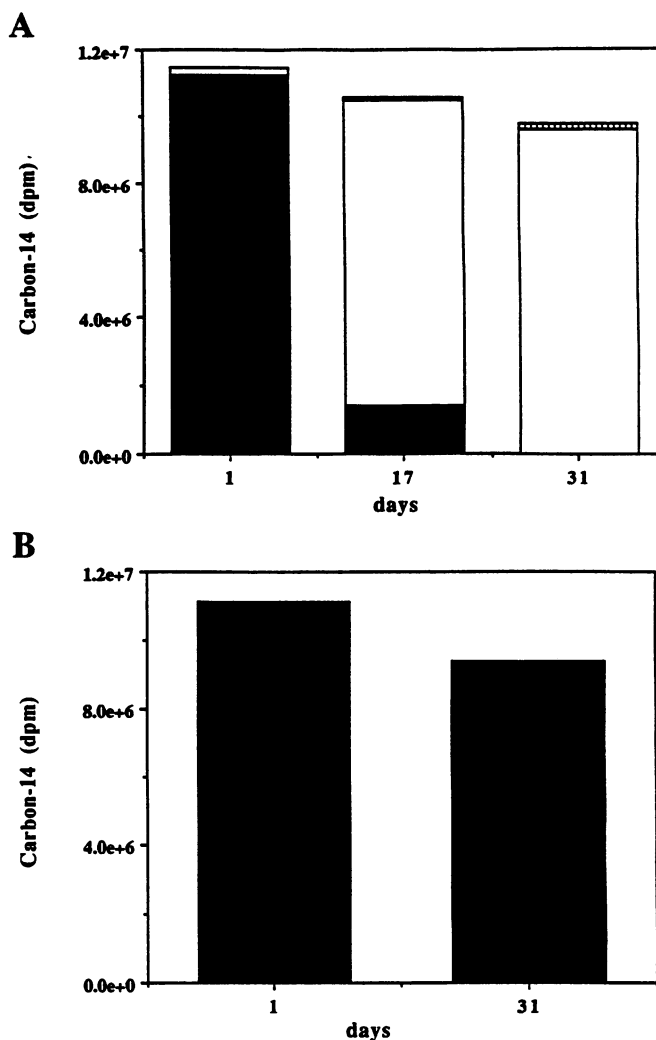


FIG. 2. Fate of ¹⁴C-labeled benzene in anaerobic microcosms. (A) Microcosm 1. (B) Sterile microcosm 6. The specific activity of benzene was 600,000 dpm/ μmol . Solid bars, volatile fraction (benzene); hatched bars, nonvolatile fraction; open bars, carbon dioxide.

Galić and Vogel (13). ¹⁴C activity was determined on a Tri-Carb model 4530 scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.). Two active microcosms and the sterile control microcosm were amended with ¹⁴C-benzene. Microcosms 1 and 6 (sterile) were spiked with 70,000 dpm/ml at an initial benzene concentration of about 95 μM . Microcosm 2 was spiked with 160,000 dpm/ml at an initial benzene concentration of about 400 μM . In microcosm 1, approximately 90% of the label from benzene was retrieved in ¹⁴CO₂ (Fig. 2A). No ¹⁴CO₂ was formed in the sterile microcosm (Fig. 2B). In microcosm 2, ca. 90% of the ¹⁴C-benzene degraded was recovered as ¹⁴CO₂, although the rate of degradation was about an order of magnitude slower than in microcosm 1, most likely as a result of the higher initial benzene concentration (data not shown).

The terminal electron acceptor for this process has not yet been established, although sulfate is a likely candidate. There was no nitrate in the sediment or in the medium, eliminating the possibility that nitrate was the electron acceptor. Some methane was produced in each active mi-

crocasm ($0.3\% \pm 0.1\%$ in the headspace); however, this amount of methane is equivalent to about 5% of the benzene actually degraded in these microcosms. In addition, the amount of CO_2 formed from benzene (90% CO_2) in the radiolabeled experiment is more consistent with sulfate reduction than methanogenesis, because during methanogenesis 62.5% of the carbon from benzene would theoretically be catabolized to methane and only 37.5% would be catabolized to CO_2 (13). The sulfate concentration in active microcosms appeared to decrease slightly relative to the sterile control, from 20.0 to 19.5 ± 0.3 mM. This change in concentration (from 20 to 19.5 mM) is consistent with the theoretical amount of sulfate that would be consumed if all of the electrons from 140 μM benzene were used to reduce sulfate to sulfide. Such small changes in sulfate concentration were difficult to measure, and hence the measured decrease in sulfate concentration was not statistically significant ($n = 4$; $P \geq 0.05$). Finally, it was demonstrated previously that sulfate was the terminal electron acceptor for toluene and xylene degradation in similar microcosms with the same source of inoculum (i.e., Seal Beach sediment) (8). Therefore, we believe that sulfate was probably the electron acceptor, although it is not possible at this time to exclude the possible role of CO_2 or oxidized metals present in the sediment as electron acceptors in the degradation of benzene. We also cannot exclude the possibility that sulfate was depleted as a result of the oxidation of unknown electron donors present in the sediment.

In this study, we have shown that a subsurface microbial community can degrade benzene under strictly anaerobic conditions. Care was taken to exclude any possible involvement of molecular oxygen in the degradation of benzene. The complete mineralization of benzene to carbon dioxide was confirmed by using ^{14}C -labeled benzene. The rates of degradation varied from 0.36 to 3.7 $\mu\text{M}/\text{day}$, depending on substrate concentration and on the presence of other carbon sources (toluene). This study stresses the importance of environmental conditions, notably the presence of alternate substrates, on the observation of metabolic activities and may help to explain the varied results seen in the literature on the anaerobic biodegradability of benzene.

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